An investigation into the relationship between intracellular bacteria and *Acanthamoeba* spp.

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Molecular Genetics in the College of Arts and Sciences of The Ohio State University

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Abstract

Acanthamoeba is an opportunistic single-celled protist, found ubiquitously in nature. It is the causative agent of the sight-threatening eye disease, *Acanthamoeba* keratitis (AK), and has been shown to act as a protective harbor for a variety of bacterial species. These pathogenic bacteria, including *Legionella*, the causative agent of Legionnaire's disease, as well as *Pseudomonas*, associated with certain nosocomial infections, may act as a source of virulence for the *Acanthamoeba* host cell.

Spanning the years 2003-2005, Chicago, Illinois experienced a dramatic increase in the incidence of *Acanthamoeba* keratitis. This rise in AK cases has been hypothesized to be a direct result of Environmental Protection Agency (EPA) mandated water treatment changes, which resulted in a greater number of microorganisms in the water. This increased level of possibly pathogenic bacterial species meant more food for those *Acanthamoeba* living in these water sources, which could have resulted in the witnessed increase in prevalence of the amoeba. It has been our goal to characterize these *Acanthamoeba*, isolated from both Chicago water sources as well as AK patients, in the hopes of detecting the presence of pathogenic intracellular bacteria, which may have contributed to the Chicago AK outbreak. To date, 50 clinical samples of *Acanthamoeba* obtained from Chicago AK patients, as well as 36 water samples from the Chicago area, have been screened for the presence of *Legionella*, *Pseudomonas*, *Mycobacteria*, and *Microbacteria* using genus specific PCR of the 16S rRNA gene.

Additionally, a portion of this thesis is concerned with a Legionnaire's disease outbreak, which took place during the summer of 2013. Our aim in this study is to ascertain the possible involvement of *Acanthamoeba* spp. in this outbreak. Twenty-one environmental water samples and 9 biofilm swab samples, which have been screened for the presence of *Legionella* by the

Center for Disease Control and Prevention (CDC) from the outbreak site, were analyzed in the attempt to culture any amoeba present. These samples were screened in our lab to confirm the presence of *Legionella* DNA via PCR amplification, utilizing genus-specific 16S rDNA primers. To date, one sample, taken from a cooling water tower, is confirmed as having *Acanthamoeba* presence via PCR using *Acanthamoeba*-specific primers of the nuclear 18S rRNA gene, followed by DNA sequencing. This same sample was both PCR and sequence confirmed as containing *Legionella pneumophila.* Current results suggest possible *Acanthamoeba* involvement in this Legionnaire's disease outbreak, and may shed light on how the outbreak was able to occur.

Acknowledgements

I would like to thank Monica Crary, for her initial work on the Chicago *Acanthamoeba* keratitis outbreak of the early 2000s. I would also like to thank our collaborator, Natalio Kozak-Muiznieks, of the Center for Disease Control and Prevention (CDC) for providing us with the swab and bulk water samples from the Legionnaire's disease outbreak site. Additionally, I would like to thank Norman Groves for all his help and insight into the uses of a confocal microscope. Lastly, I would like to thank the Ohio State's Arts and Sciences Honors Committee for their generous Undergraduate Research Scholarship, without which this project would not have been possible.

I dedicate this senior thesis:

To my parents, Linda and Andy, and my siblings, Kate, Daniel, and Alai, for their unwavering support, and feigned comprehension at what I do in the lab;

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Chapter 1

Introduction

What is *Acanthamoeba*?

The protist *Acanthamoeba* is an opportunistic, mitochondriate, unicellular Free-Living Amoeba (FLA), found ubiquitously in nature. *Acanthamoeba* has been isolated from soil, dust, air, sea water, domestic and industrial water sources, as well as from animal and human tissue samples[52]. Many *Acanthamoeba* species are known to act as hosts for several pathogenic bacteria, potentially contributing to the virulence of amoeba infections. The genus derives its name from the Greek morpheme "acanth", meaning "spiky" or "spiny", as a tribute to the amoeba's acanthopodia – the small, thorn-like "fingers" that form along the surface of its body[81]. *Acanthamoeba* is able to mobilize by means of its pseudopods, which quite literally translate from Greek to "fake feet"[44].

Acanthamoeba is a curious organism, and has been used as a model organism for a variety of cellular analyses. First, it is a model organism for cellular movement and the cytoskeleton. Amino acid alignment of *Acanthamoeba* and vertebrate actin polypeptides shows a 95% sequence similarity between the two, rendering *Acanthamoeba* a viable model system for studying cytoskeleton structure in chordates. Second, owing to *Acanthamoeba*'s ability to house a vast array of microorganisms as intracellular bacteria or as potential endosymbionts, it is an informative model for symbiont studies and gene transfer questions. Lastly, as will be discussed more fully in subsequent sections, extensive analysis has been done on *Acanthamoeba* genotypes and classification. Ergo, the protist is an excellent model organism for phylogenetic analysis and evolution.

Analysis of *Acanthamoeba*'s rRNA sequences has resulted in an estimate of the protist having diverged sometime between the divergence of yeast (approximately 1.2 billion years ago) and the divergence of plants and animals (approximately 1.0 billion years ago). Furthermore, *Acanthamoeba* is considered to be an amphizoic amoeba, owing to its ability to exist both as a free-living organism, as well as a parasite within host cells[57].

Notably, *Acanthamoeba* has a biphasic life cycle, as its two morphological forms occur as active trophozoites, and dormant cysts. Historically, *Acanthamoeba* species have been classified based on the morphology of their trophozoites as well as the size and morphological characteristics of their cysts. This classification method resulted in the *Acanthamoeba* genus being divided into 25 different species, based solely on cellular morphology[15]. Modern classification systems now rely on molecular methods, using the nuclear 18S ribosomal RNA gene, to place *Acanthamoeba* species into specific groups. There exist at least 18 T-groups, designated T1-T18, based on a 5% divergence threshold after standard sequence alignment of the 18S rRNA gene.

The History of *Acanthamoeba*

Acanthamoeba was first discovered by Castellani in 1930. The amoeba was intermingled in a culture of the fungus *Cryptococcus pararoseus*. It was distinguishable by its round shape, and was found to have a diameter of 13.5 µm to 22.5 µm. Castellani observed pseudopodia on the surface of the trophozoites, and noted cysts as double-walled in morphology, with an average diameter of 9 µm to 12 µm. Due to these characteristics, Castellani placed the amoeba in the genus *Hartmannella*. Following the amoeba's discovery, in 1931 Volkonsky subdivided the genus *Hartmannella* into three distinct genera – *Hartmannella*, consisting of those amoeba with

round, smooth walled cysts; *Glaeseria*, consisting of those amoeba whose cysts can perform nuclear division; and *Acanthamoeba*, consisting of those amoeba whose morphology included a double-walled cyst with an irregular outer layer, and the presence of pointed spindles at mitosis[81].

Following years of debate on the authenticity of the genus *Acanthamoeba*, and final reaffirmation of its integrity, *Acanthamoeba* was discovered as a contaminant in a sampling of tissue culture in the late 1950s[16, 39]. Subsequently, in 1958-1959, Culbertson et al. demonstrated *Acanthamoeba*'s pathogenic nature, in having the capacity to cause cytopathic effects on monkey kidney cells in vitro, as well as having the ability to kill laboratory animals when inoculated[44].

The following two decades resulted in the first reported and characterized cases of two diseases, both of which *Acanthamoeba* is the causative agent: in 1972, the first reported case of Granulomatous Amebic Encephalitis (GAE) was observed[69]; the following year, 1973, the first reported case of *Acanthamoeba* keratitis (AK) was witnessed[44]. Importantly, in 1975, Proca-Ciobanu *et al*. reported on *Acanthamoeba*'s inherent ability to act as a harbor for intracellular bacterial endosymbionts. Furthermore, as will be discussed in later sections, Krishna-Prasad and Gupta showed *Acanthamoeba*'s ability to house the bacterial genus *Mycobacterium*, in their 1978 study, and in 1980, *Acanthamoeba* was linked to Legionnaire's disease[62].

Where is *Acanthamoeba* found?

Acanthamoeba is a highly ubiquitous organism, which has been isolated from a countless number of locations throughout the years it has been studied. From natural settings, *Acanthamoeba* has been isolated from the surface of sea water, fresh water, vegetation, soil, and a variety of body (both human and animal) surfaces and tissue samples. From manmade settings,

Acanthamoeba has been isolated from factory discharges, water reservoirs, storage tanks, hospital settings, drinking water, and bottled water[44]. It is important to highlight the ability of *Acanthamoeba* to inhabit a wide array of environments and conditions, in emphasis of the amoeba's ability to infect a variety of locals. This capacity to infect stems from *Acanthamoeba*'s high tolerance for extreme environments, including high osmolarities, temperatures, pressures, and chemical treatments.

Further confirmation of *Acanthamoeba*'s ubiquitous presence in nature was shown in a 1980 study by Curson *et al.*. This work sought to demonstrate the mass common exposure we as humans have to this protist. Curson *et al.* analyzed normal human sera from asymptomatic individuals in New Zealand, and found that, of those individuals scored for the presence of anti-*Acanthamoeba* antibodies, 100% were positive in varying degrees of antibody concentration[17]. The human immune system produces these antibodies upon infection by the microorganism in question, thereby confirming contact to some degree, regardless if any disease or visible infection occurs.

Genotyping

At the onset of *Acanthamoeba*'s discovery and research, species names within the genus were attributed to the individual who discovered the novel species, the location from which the isolate was obtained, or a variety of other criteria, with differentiation between species being due solely to morphology. As a result, a vast array of isolates existed in desperate need of categorization. Thus, as modern molecular mechanisms were non-existent in the 1970s, scientists of the time categorized isolates based upon the morphological characteristics of the cysts. This classification system resulted in eighteen species categorized into three groups[60] (**Table 1**).

	Group 1	Group 2	Group 3
Group Specifications	Large trophozoites, and cyst forms have widely separated endocysts and ectocysts	Cyst diameter is less than 18 µm, and cyst shape may be polygonal, triangular, or round; ectocyst may be either thick or thin	Thin ectocyst, and mean cyst diameter is less than 18 µm
Species	*A. stronyxis, A. comandoni, A. echinulate, A. tubiashi*	*A. mauritaniensis, A. castellanii, A. polyphaga, A. quina, A. divionensis, A. triangularis, A. lugdunensis, A. griffin, A. rhysodes, A paradivionensis, A. hatchetti*	*A. palestinensis, A. culbertsoni, A. royreba, A. lenticulata, A. postulosa*

Table 1*: Early categorization of the genus Acanthamoeba, based solely upon morphological characteristics and differences.*

While this classification system may have been useful at the time, grouping via morphology leads to inconsistencies, as the morphology of *Acanthamoeba* trophozoites and cysts vary with culture conditions[60]. Modern techniques for genotyping now rely on molecular analysis for classification. The late Dr. Thomas Byers of The Ohio State University began work in the 1980s on categorizing the isolates in the genus *Acanthamoeba*, based on rRNA gene sequences. Relying upon both nuclear 18S rDNA and mitochondrial 16S rDNA, it is now evident that there exist at least eighteen different genotypic classes, designated T1-T18. Each T group is separated from one another by at least 5% sequence divergence[65].

Acanthamoeba's role in the environment

Recent work has shown *Acanthamoeba*'s importance in the biogeochemical cycle and the microbial loop. Organic material is consumed by bacteria, which are subsequently ingested by environmental *Acanthamoeba*. The amoeba is then ingested by larger, multicellular organisms, which in turn produce organic waste, and the cycle begins anew. As further proof of *Acanthamoeba*'s importance in the ecosystem, soil that contains both *Acanthamoeba* and

bacteria contains a much higher level of mineralization of carbon, nitrogen, and phosphorous, when compared to soil samples that contain bacteria but no *Acanthamoeba*[44].

Biphasic Life Cycle

Trophozoites

Acanthamoeba exists in its trophozoite form only in favorable conditions, i.e. correct pH, temperature, as well as availability and type of nutrients[52]. Depending upon the species of *Acanthamoeba*, these trophozoites can range in size from 15 µm to 50 µm, with visible acanthopodia on the surface of its body (**Figure 1**). It is in the trophozoite stage that *Acanthamoeba* have their characteristic "spiky" appearance. When viewed under 1000x magnification, the nucleus, mitochondria, and vacuoles, are clearly visible. As a trophozoite, the amoeba has the ability to replicate via binary fission, eat via phagocytosis, and grow. The word trophozoite comes from the Greek word, "tropho", meaning "to nourish", reflecting the activity of the organism while in this stage.

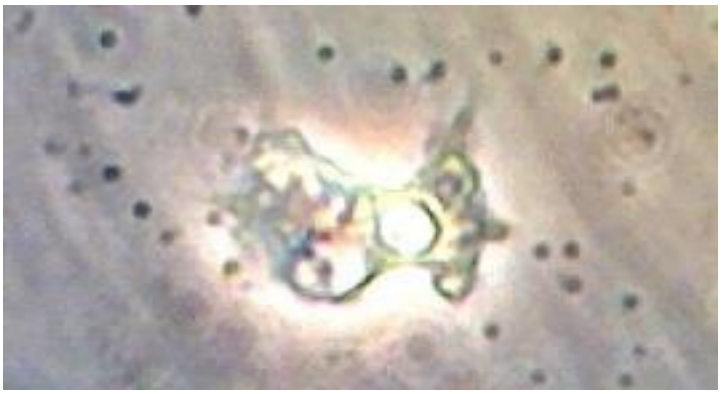

***Figure 1**: When Acanthamoeba is in its active, feeding, trophozoite stage, spiky acanthopodia are visible on the surface. The nucleus is also visible, along with mitochondria, and food vacuoles. Note the characteristic autofluorescence as well.*

Cysts

Acanthamoeba's most noteworthy morphological characteristic is its ability to form dormant, resistant, cysts. When environmental conditions are unfavorable, such as poor nutrient

availability, extreme pH, temperature, or a variety of environmental conditions, the amoeba will undergo encystment. In this form, the *Acanthamoeba* cyst is double-walled - the outer wall, the ectocyst, surrounds the inner wall, the endocyst[81] (**Figure 2**). While the typical body shape of a trophozoite is "spiky", that of a cyst takes on a more spherical or polygonal shape, lacking its trophozoite-counterpart's acanthopodia. Cysts are similarly uninucleate, and appear to lack mitochondria[75].

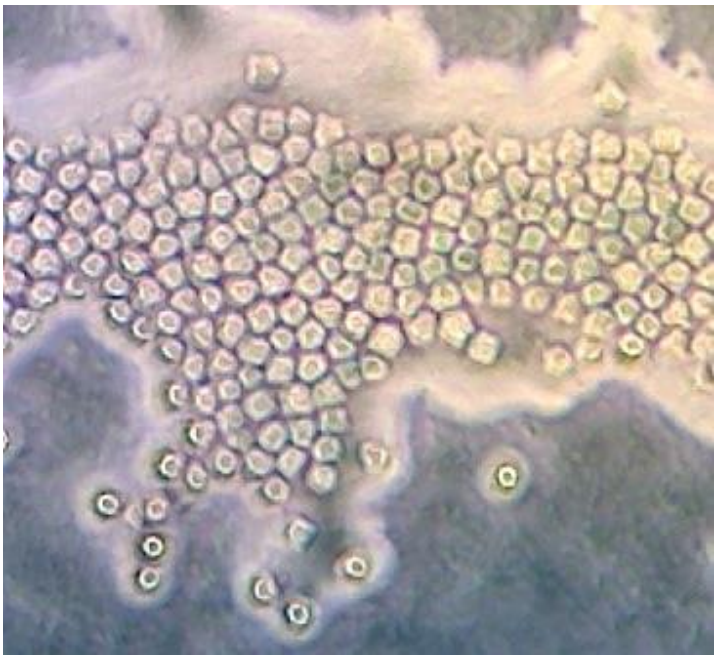

***Figure 2**: A clustering of Acanthamoeba cysts. Acanthamoeba will encyst when environmental conditions can no longer support Acanthamoeba growth and survival. In this stage, the amoeba has minimal metabolic activity, and is neither growing nor reproducing.*

It has been demonstrated, by both our lab as well as others, that *Acanthamoeba* may remain encysted, and still viable, for greater than twenty years. It is during this time that the amoeba exhibits minimal metabolic activity, and there is a substantial decline in the cellular levels of RNA, proteins, triacylglycerides, and glycerol. On the other hand, there is a substantial increase in the rate of synthesis of cellulose and acid-insoluble protein-containing material. It is also this stage of the *Acanthamoeba* life cycle that has been found to be resistant to a variety of treatments and environmental factors, including chlorine treatment[5]. As an additional note, both trophozoite and encysted *Acanthamoeba* express a characteristic autofluorescence, which aids in finding both forms in culture with other microorganisms.

Acanthamoeba Feeding Habits

While in its trophozoite stage, *Acanthamoeba* is known to feed on yeast, other protists, bacteria (both gram-negative and gram-positive), small organisms, and organic particles[44]. The microorganisms and small particles *Acanthamoeba* ingests tend to be found on the surface of its habitat, mainly at the air-water interface of aquatic environments[8, 59]. The amoeba utilizes its acanthopodia to capture these food particles, and relies upon phagocytosis and pinocytosis for ingestion.

When growing *Acanthamoeba* in culture, there is an ideal bacteria-to-amoeba ratio that must be reached in order for the protist to grow successfully. This ratio has been showed to be approximately 10:1 bacteria to amoeba. The specific balance that must be reached is two-fold in necessity: first, the bacteria, typically *E. coli*, must be abundant enough to support *Acanthamoeba* growth; second, too many bacteria in culture with amoeba will overrun the growth of the amoeba, ultimately inhibiting growth due to a lack of physical space[52].

Disease

Genotypic analysis of disease-causing *Acanthamoeba*

As stated previously, there exists a vast array of *Acanthamoeba* species. Of particular interest for our study are those isolates to be included in groups T3, T4, and T11. It has been shown that *Acanthamoeba* within these three T groups, especially those of T4, provide the vast

majority (>90%) of those species associated with *Acanthamoeba* keratitis (AK)[71](**Figure 3**).

Acanthamoeba genotypes in groups T1, T10, and T12, have been isolated from infections not related to AK, the latter two having never been found in the environment[4].

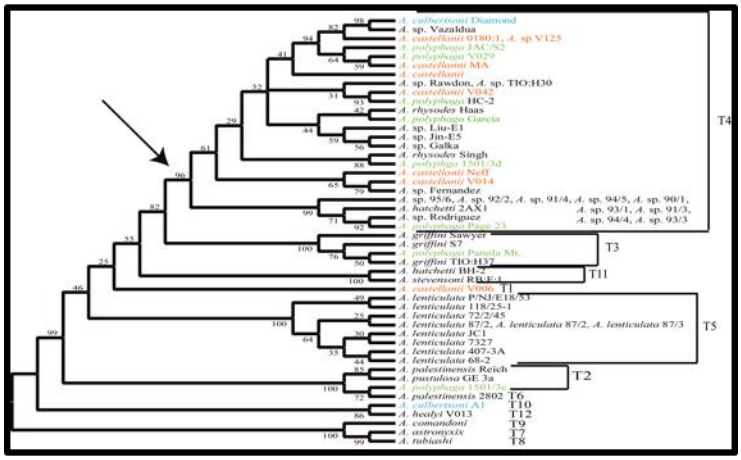

***Figure 3**: Acanthamoeba genotype phylogenetic tree. While historical classification has mainly relied on morphological differences between species, modern molecular approaches utilize utilize divergence within the 18S rDNA gene to classify Acanthamoeba genotypes.*

An essential aspect for infection and disease by *Acanthamoeba* is physical contact, which is mediated by a 130 kDa Mannose-Binding Protein (MBP) found on the surface of *Acanthamoeba* trophozoites[25]. The gene encoding this protein is comprised of six exons and five introns, the sequence of which spans 3.6 kb of the *Acanthamoeba* genome, and whose unedited protein product contains 833 amino acids[68]. The initial binding of *Acanthamoeba*'s MBP to host-cell surface mannose-containing glycoproteins results in the host-cell phagocytosing the amoeba. This uptake of *Acanthamoeba* into a host-cell causes the amoeba to release toxins that result in host cell death via the PI3K pathway[68].

Additionally, a major mechanism by which *Acanthamoeba* may induce apoptosis is by a resultant influx of calcium into the host-cell cytosol upon initial *Acanthamoeba* binding onto the surface of the host-cell. This binding of *Acanthamoeba* alters the host-cell's own signaling machinery, resulting in an uptake of external calcium. This influx, in turn, causes a morphological change in the cytoskeletal structure of the host-cell, and can alter the permeability of its plasma membrane, resulting in target cell death[73, 68, 44]. In the 1990s, investigators

hypothesized that, as phagocytosis is an actin-dependent process, there must be a relationship between the role of *Acanthamoeba* in host cell death, and that of the viability of the host cell's actin. Investigators used the drug Cytochalasin D, a toxin known to inhibit the polymerization of actin monomers, and showed that, without actin polymerization, *Acanthamoeba*-mediated host cell death is inhibited[54, 73]. This demonstrated that actin-mediated cytoskeletal rearrangements play an important and necessary role in the pathogenesis of *Acanthamoeba* infection.

Immune Response and Defense

As with any response induced by a microorganism, there exists, in the human defense system, a primary response and a secondary response. The primary, or non-specific innate immune response, begins with the basic physical barrier to infection – skin cells. Skin cells produce and secrete fatty acids, lysozymes, and other chemicals that have the capacity to break down cells walls of possible pathogenic invaders, and have anti-microbial properties[44]. If this initial barrier fails, *Acanthamoeba* invasion into host tissue may occur (**Figure 4**). When this happens, components of the immune response will secrete cytokines

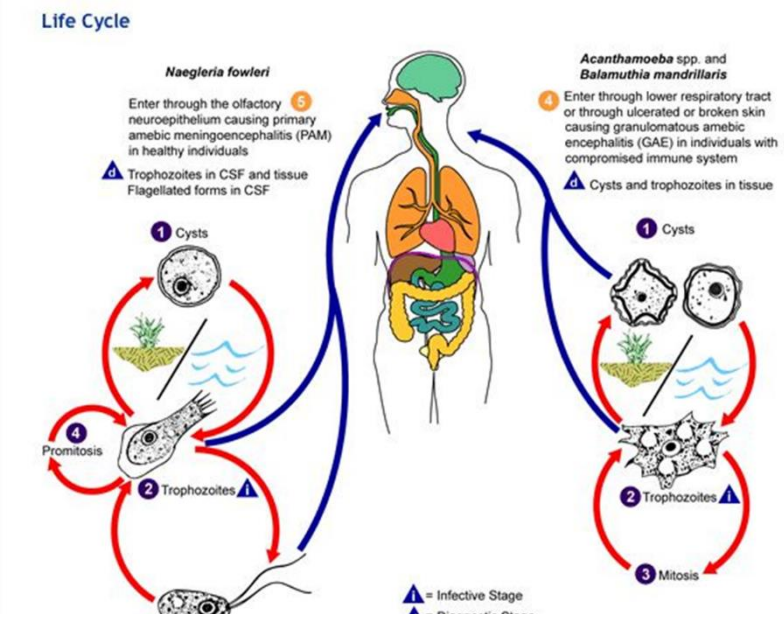

***Figure 4**: Life cycle of disease-causing Acanthamoeba. Taken from the Center for Disease and Control website.*

in order to recruit additional members of the immune response, and subsequent formation of antibodies targeted to the invading antigen. If infection were to occur at a later point by the same antigen, this would result in the activation of the secondary immune response, in a quicker and more effective manner owing to the already present targeting antibodies.

Granulomatous Amebic Encephalitis (GAE)

Granulomatous Amebic Encephalitis (GAE) is a tragic, almost universally fatal, *Acanthamoeba* infection of the brain and Central Nervous System (CNS). It is caused by a subset of *Acanthamoeba* species, namely *A. culbertsoni, A. castellanii, A. polyphaga, A. astronyxis, A. healyi, and A. dicionensis*[81]. Interestingly, the majority of *Acanthamoeba* isolates associated with GAE are from group T4, suggesting that there may be a common underlying feature of these species that gives them a heightened capacity to infect. Or, more simply, T4 is the most common genotype of *Acanthamoeba*, found in all *Acanthamoeba* infections, and is dispersed widely in the environment. The symptoms of GAE typically begin as headaches, a stiff neck, abnormalities in one's mental state, and can later progress into nausea, vomiting, fatigue, sight-impairment, fever, uncoordinated movements, hemiparesis, seizures, coma, and eventually, death. Facial palsy resulting in facial asymmetry is also experienced on occasion[80]. GAE is a slow-developing disease, which can span for weeks to several months before symptoms are experienced[57]. *Acanthamoeba* is known to infect more severely the cerebral hemispheres of the brain as compared with other regions of the CNS, and results in severe hemorrhagic necrosis[67].

GAE has been found most typically in those with already compromised immune systems, although this is not universally a necessity. Specifically, those who have been diagnosed with HIV/AIDS, diabetes, or have undergone organ transplantation have a higher susceptibility to the disease[81]. That being said, GAE has also been found in the CNS of a number of animals, including gorillas, monkeys, dogs, ovines, bovines, horses, kangaroos, birds, reptiles, amphibians, fish, and invertebrates[80, 20, 79].

Diagnosis of GAE can be performed either by laboratory diagnostic methods or by neuroimaging. In the lab, a biopsied tissue sample will contain *Acanthamoeba* cysts, which can be easily viewed under a microscope. Subsequent Immunofluorescent Assay (IFA), Indirect Immunofluorescence (IIF), and/or Polymerase Chain Reaction (PCR) are also used to confirm the presence of *Acanthamoeba* in these tissue samples. If neuroimaging techniques are used, such as a CT or an MRI, the presence of "space-occupying or ring-enhancing lesions" will be evident[81].

Treatment of GAE is aimed at killing its causative agent, *Acanthamoeba.* The drugs ketoconazole, miconazole, 5-flucytosine and pentamidine have been shown to be successful at killing *Acanthamoeba in vitro*, and an early diagnosis may result in successful treatment should these anti-amoeba drugs prove viable *in vivo*[82].

Acanthamoeba keratitis (AK)

Acanthamoeba keratitis (AK) is a painful eye disease that threatens visual impairment of the individual who has contracted the affliction. In AK, the corneal tissue becomes inflamed, and symptoms include the excessive production of tears, eye-redness, abnormally low "drooping" of the upper eyelid (eyelid ptosis), an increased blood flow to the conjunctival blood vessels (conjunctive hyperemia), and excessive sensitivity to light (photophobia) (**Figure 5**). Glaucoma has also been shown to be reported alongside AK as well[44]. While the onset of these symptoms appears to be highly variable, which may be due to the varying strength of the immune system of the patient or due to the extent of corneal trauma, symptoms may arise following anywhere from a few days to several weeks post-infection[44]. Additionally, as *Acanthamoeba* is well-known to harbor intracellular bacteria, secondary infection due to bacterial invasion may additionally complicate the pathogenicity of the disease. AK has not been associated to occur in higher levels in those who are immunocompromised.

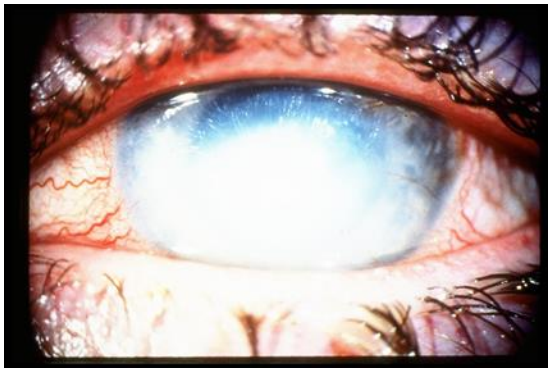

***Figure 5**: Individuals who contract AK experience severe eye pain, in addition to vision impairment, and the characteristic stromal ring.*

The first reported case of AK occurred in 1973, when a link was made between an individual with persistent ocular trauma and his exposure to contaminated water.[44] Since then, a great deal of work has gone into the etiology of AK cases across the world. On a cellular level, AK is the result of *Acanthamoeba* attachment to the cornea by means of the protist's MBP. This interaction results in the breakdown of the epithelial barrier, invasion into the stroma, depletion of host tissue keratocytes, induction of a severe response by the immune system, and, eventually, necrosis[26, 78]. It is necessary to note that as the MBP appears to be necessary for *Acanthamoeba*

invasion of stromal tissue, the *Acanthamoeba* must be present in the trophozoite form for successful invasion to occur[19, 24]. Additionally, *in vivo* studies have shown that animals with intact corneas do not develop AK, indicating that some sort of lesion on the cornea is necessary for AK to occur[54].

Recent studies have shown there to be a positive correlation between the modern practice of wearing contact lenses and the incidence rate of AK[5]. While *Acanthamoeba* have been shown to attach onto both the antiquated "hard" lenses as well as the more modern "soft" lenses, *Acanthamoeba* shows a definite preference for soft contact lenses[44]. It is also interesting to note that *Acanthamoeba* also preferentially adheres to those contact lenses that have been previously worn, as opposed to those which have not. Contact lenses that have been previously worn contain a variety of saccharides, including mannose, which may facilitate the binding of the *Acanthamoeba* via its MBP[30, 47, 77]. In this sense, the modern contact lens acts as a vector by which *Acanthamoeba* are carried from contaminated water onto the surface of one's eye.

An additional point of credence to the association between the popularity of contact lens use and the incidence rate of AK is the commonality in which biofilm forms either on the contact lens itself, or forms in the contact lens storage case. Biofilm, any group or collection of microorganisms that stick to one another on a surface, is the foodstuffs of *Acanthamoeba*. Thus, the presence of biofilm in contact lens cases and on contact lenses may result in an increase in the attachment affinity for *Acanthamoeba* as well as promote the growth and survival of the protist[44].

Diagnosis of AK involves corneal scrapings and biopsy, and analyzing the isolates for the presence of *Acanthamoeba* cysts[80]. AK patients, in addition to having the aforementioned symptoms, also display 360 degree paracentral stromal ring, visible to the naked eye, a result of

the continuous breakdown of corneal epithelium, and is characteristic of the disease. Usually, infection will only be unilateral, although bilateral keratitis has been diagnosed. Typically, the only way to treat AK is through combination therapy of two or more biocides, occasionally with antibacterial properties, depending on the characteristics of the infection[48]. If the disease has spread deep enough, surgery is necessary – corneal transplants are often the end result of an AK infection.

Preventative measures are available, even for those who are consistent contact lens wearers. Proper lens care and hygiene are essential for correct lens maintenance. As *Acanthamoeba* are particularly prevalent in larger bodies of water, both manmade and naturally occurring, individuals should avoid wearing contact lenses while swimming, performing in water-sports and activities, while in a hot tub, or in the shower. Similar care should be taken with the contact lens case as well, ergo discarding solution after each use, and consistently sanitizing the case itself.

Endosymbionts

It is a well-established fact that *Acanthamoeba* are excellent at harboring intracellular bacteria. Approximately 20-24% of *Acanthamoeba* obtained from both clinical and environmental settings contain intracellular bacteria, as well as other microbial endosymbionts[21]. To date, the vast array of intracellular bacteria isolated from *Acanthamoeba* include *Francisella tularensis, Mycobacterium avium, Burkholderia spp., Vibrio cholera, Listeria monocytogenes, Heliobacter pylori, Afipia felis* and *Escherichia coli* [29, 2]. Furthermore, 5% of *Acanthamoeba* isolates have been found to contain *Chlamydia, Chlamydophila,* and *Chlamydia*-like bacteria[21]. It has been hypothesized that this practice of maintaining intracellular bacteria increases the virulence of *Acanthamoeba.* While this has not been universally proven, a 1997 study concluded

that *Acanthamoeba* that had been infected with *M. avium* are more pathogenically aggressive than non-infected amoeba in a mouse model[12]. It has also been shown that this may increase the virulence of particular bacterial species, as *Legionella* that have passed through *Acanthamoeba* have been shown to be more aggressive than those that have not been harbored as intracellular pathogens[13].

Symbiosis

In cellular and evolutionary biology, there is a long-standing debate on the relationship held between *Acanthamoeba* and its intracellular bacteria. Historically, it has been proposed that this relationship is one analogous to a strict predator-prey interaction. In this model, *Acanthamoeba* is the only beneficiary of the relationship, in that the protist feeds on the bacteria, and the bacteria receives no benefit from the interaction[61]. While this relationship was being examined in the early 2000s, it was noted that *Acanthamoeba* preferentially feed on non-spore-forming bacteria[44]. Thus, the hypothesis emerged that, noting the poor ability of non-spore-forming bacteria to survive in harsh environments, these bacteria may have had an evolutionary necessity to associate with a host organism – for protection in harsh conditions.

This hypothesis was given credence by the 1980 study by Rowbotham, demonstrating that *Legionella pneumophila* can successfully live inside *Acanthamoeba*, avoiding degradation by *Acanthamoeba*'s digestive mechanisms. This relationship suggests *Legionella* and *Acanthamoeba* may have a long, co-evolutionary history that was able to result in the bacteria avoiding degradation, in addition to the implications this now holds for *Acanthamoeba* as a

vector for pathogenic bacteria, a possible source of "superbugs", and the transmission of said superbugs from the environment to humans. In this sense, the amoeba acts as a sort of "Trojan Horse", as many of the bacterial endosymbionts of *Acanthamoeba* are human pathogens, e.g. *E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Vibrio cholera*[44].

Legionella pneumophila

Legionella pneumophila is an aerobic, gram-negative, rod-shaped bacterium[66] (**Figure 6**).

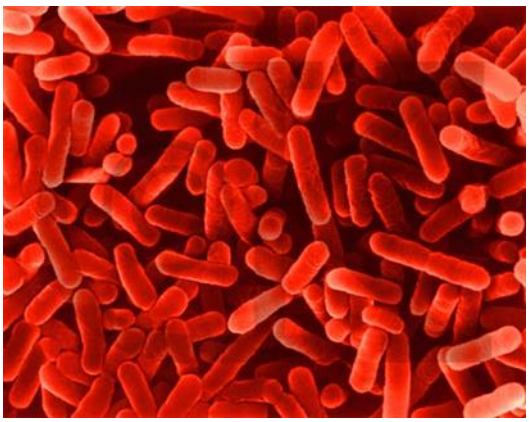

***Figure 6**: Legionella*

It is a ubiquitous, aquatic bacteria, and an obligate intracellular pathogen that infects a variety of diverse, water-residing amoeba and protists[56]. Additionally, *Legionella* is most typically isolated from large bodies of water, both natural and manmade, including lakes, streams, and cooling towers[7, 18, 53]. While the bacteria later named *Legionella* was first discovered in 1943, the bacteria's role in causing disease was not initially known[49].

The *Legionella* genome is comprised of a single circular chromosome, approximately 3.3 million bp in length, and the genome contains approximately 3,000 genes[27]. The *Legionella* genome encodes for a number of eukaryotic-like proteins, which may reflect the bacteria's ability to infect human and animal host cells[27].

Acanthamoeba Phagocytosis of *Legionella*

Acanthamoeba tend to ingest their food rather rapidly, as within 40 seconds, a bound particle may be surrounded by the amoeba's pseudopods in a process known as "coiling

phagocytosis", taken into the cytoplasm, and released intracellularly as a phagosome[44]. Typically, during ingestion, these phagosomes fuse with a lysosome containing digestive enzymes[32]. However, *Legionella* is unique in its uptake, as the latter fusion step is avoided. When *Legionella* is taken up into its host cell, it is contained in a specialized vacuole, creatively named the "Legionella-Containing Vacuole", or LCV[1]. *Legionella* blocks its own degradation by secreting more than 250 effector proteins into the host cell, which are able to hijack the host cell's digestion machinery, and block the fusion of the lysosome and the LCV[34, 33, 35].

When this inhibition of degradation occurs, the bacteria are able to, not only survive, but proliferate within its protective host cell. This provides the opportunity for, not only lysis of the amoeba at a spatial threshold level of bacterial proliferation, but bacterial evolution in its protective microenvironment[23]. It also appears that this capacity to avoid degradation while inside *Acanthamoeba,* especially during encystment, is characteristic of *Legionella* alone[50, 74, 12, 70].

Legionnaire's Disease

The first reported case of Legionnaire's disease (LD) occurred in 1976. On July 21st-24th, the annual convention of the American Legion's Pennsylvania Department was being held at the Bellevue-Stratford Hotel in Philadelphia. Of the 4,000 members present, along with delegate-family members, 221 individuals developed what was initially termed a "mysterious illness", comprised of fever, cough, and fluid-filled lungs. Of the 221 individuals who contracted the illness, 34 passed away. The Center for Disease Control and Prevention (CDC) was called in to investigate, and discovered the previously unnamed affliction – Legionnaire's disease[72].

Through the course of their investigation, the CDC was able to determine the causative agent of LD. Inoculation of guinea pigs with lung tissue cuttings that had been obtained from patients who had passed away from the Philadelphia outbreak resulted in the isolation of a previously undescribed bacterial species, which was subsequently named – *Legionella pneumophila*. Thus, although the bacteria itself was not of novel discovery, as *Legionella* had been isolated more than three decades prior, the CDC was now able to classify the gram-negative bacteria as one of pathogenic, disease-causing nature. Further confirmation that this was indeed the causative bacterial agent of the outbreak occurred about six months later, in the McDade laboratory. He was able to confirm the presence of *Legionella* antibodies in the serum of those who had survived the outbreak, thus showing all those individuals who had contracted the "mysterious illness" had been infected with the same bacterial species[84]. Thus, the initial classification of Legionnaire's disease was, "an acute, febrile, respiratory illness that primarily affected member's and visitors attending the 58th annual convention of the Pennsylvania American Legion"[49].

Modern Classification of Legionnaire's Disease

The symptoms of Legionnaire's disease are similar to those found in other types of bacterial pneumonia, the onset of which is typically anywhere from two to eleven days following exposure[9, 11]. The early symptoms of LD typically consist of malaise, headache, and general overall muscle pain. Within two days, the affected individual will begin to develop chills as well as a fever. As *Legionella* also may infect the CNS, those symptoms include general clumsiness, ataxia, slurred speech, and overall confusion[9, 11]. After about four to seven days after inoculation,

nonproductive cough and dyspnea occur. Following these symptoms in the lower respiratory tract, pulmonary symptoms may occur, and can progress the disease to life-threatening pneumonia[11].

Legionnaire's disease is known to be transmitted via the aerosolization of contaminated water, and initial invasion happens by inhalation of this contaminated steam and subsequent infection of human alveolar macrophages[56]. LD has also been found to infect people of all ages[72]. It is, however, found to be more common in those who are immunocompromised, especially individuals who have been diagnosed with HIV/AIDS.

Diagnosis

Modern diagnostic methods for detecting Legionnaire's disease typically involve a chest x-ray and/or a physical exam of the patient. Commonly, clinical physicians will use a urine test to detect the presence of *Legionella* antigen. Similarly, physicians may use blood instead of urine to determine the presence of *Legionella* antibodies, which will be present either shortly after infection or shortly after recovery. Doctors may also obtain either a sample of phlegm from the patient, or a lung tissue sample, and attempt to grow out in culture any *Legionella* that may be present[10]. Legionnaire's disease can typically be successfully treated with antibiotics specific for *Legionella*, although lung failure may still occur rarely. The mortality rate of this illness is 5-30%, as reported by the CDC[76].

Chapter 2

Chicago *Acanthamoeba* Keratitis Outbreak of 2003-2005

In the early 2000s, beginning in 2003 until 2005, the Chicago area of Illinois experienced a severe *Acanthamoeba* keratitis outbreak. During this time, there were a total of 55 cases of AK identified. There were two main hypotheses surrounding the Chicago AK outbreak, specifically regarding the *Acanthamoeba* themselves. Initially, it was suggested that a novel strain of the amoeba may have been involved, citing the possibility of additional virulence through mutational change. However, genotypic analysis via PCR amplification of the partial small subunit ribosomal DNA (ssu rDNA) gene sequence showed this not to be the case. Of the 17 isolates Booton et al. (2009) sequence analyzed, all 17 were found to be of high sequence similarity to genotypes T3 and T4, both of which have been previously shown to be involved in disease. Thus, it was clear that there was no novel strain of *Acanthamoeba* taking part in the Chicago AK outbreak.

The subsequent hypothesis was that the underlying cause was a recent change in water treatment standards by the Environmental Protection Agency (EPA). The EPA, in the hopes of reducing carcinogenic byproduct in public water supplies, sought to reduce the amount of chlorine used in these water supplies as a disinfectant. However, this reduction led to a substantial increase in the amount of biofilm within these water sources, and as *Acanthamoeba* is known to feed on biofilm, this provides validity to the second hypothesis[41]. In other words, it was the overall exponential increase of *Acanthamoeba* in these water supplies that led to the AK outbreak. As *Acanthamoeba* feeds on biofilm, comprised of a multitude of, possibly pathogenic,

bacterial species, the resultant biofilm increase following the EPA change would allow for an increase in the colonization of the amoeba, owing as a result of the increased level of nutrients in the water. This also brings into play the opportunity for amoeba ingestion of possibly pathogenic bacterial species and other microorganisms, and for *Acanthamoeba* virulence to be affected by its endosymbionts.

The Role of Intracellular Bacteria

This implication for the role of intracellular bacteria in the Chicago AK outbreak was initially investigated by a graduate student in our lab, Monica Crary. Based upon previous literature, she chose to examine *Acanthamoeba* taken from corneal scrapings of AK patients as well as *Acanthamoeba* samples obtained from Chicago water supplies, and screened these samples for the presence of *Legionella* and *Pseudomonas*, via PCR using genus-specific primers of each respective 16S rRNA gene[15]. Of the 56 clinical samples she analyzed, 43% were positive for *Legionella*, 48% were positive for *Pseudomonas*, and 36% were positive for both bacterial genera. These data were compared against 38 *Acanthamoeba* samples from outside the Chicago area as a control, and of these samples, 13% were positive for *Legionella*, 16% were positive for *Pseudomonas*, and 8% were positive for both. A chi-squared statistical analysis done by Crary confirmed there to be a statistically high prevalence of intracellular bacteria in the Chicago *Acanthamoeba* samples ($p<0.001$). Additionally, fluorescent *in situ* hybridization (FISH) showed to confirm that these bacterial genera were physically residing within the *Acanthamoeba*, specifically in cytoplasmic vacuoles within the *Acanthamoeba* trophozoites. Furthermore, Crary showed that *Legionella* can reside within *Acanthamoeba* cysts for up to 6 years, and that,

conversely, *Pseudomonas* is only viable within younger amoeba, and cannot be cultured after more than one year in storage.

Expanding upon previous work

It was our aim to expand upon Crary's work in determining the prevalence of intracellular bacteria in the Chicago *Acanthamoeba* samples. In continuation of her research, we began screening for the presence of *Mycobacterium spp.*, as previous work by the Alfonso lab has shown *Mycobacterium* to be a viable bacterial endosymbiont of *Acanthamoeba*, using reportedly genus-specific primers of the *Mycobacterium* 16S rRNA gene and PCR amplification[37].

Initially, we were only screening for the presence of *Mycobacterium* in our *Acanthamoeba* samples. However, the consistent presence of a second band of a slightly larger size than our *Mycobacterium*-positive sample indicated there may be an additional bacterial species present in a subset of isolates, which was being amplified by the *Mycobacterium* primers. After contamination was ruled out, this prompted band-isolation and sequence analysis to determine the identity of this mysterious band. Sequence analysis showed this second band to be *Microbacterium*, an entirely different bacterial genus, but with enough sequence similarity in the 16S flanking regions to be amplified by our primers. Once this band's identity was confirmed, we continued screening as before, now additionally searching for the presence of *Microbacteria.* We considered this data to be important, as while it was not initially included in our search, it still may provide insight into the etiology of the Chicago AK outbreak, as *Microbacterium spp.* have been found in eye infections in other lab studies[85].

Materials and Methods

In this study, we used the same samples utilized by Crary et al.[15] (**Figure 7**). Thirty-six *Acanthamoeba* samples were derived from Chicago water samples, and 50 *Acanthamoeba* samples were derived from Chicago AK patients. DNA was extracted from their respective source using the DNeasy Kit. Our PCR protocol was as follows: standard reagents were used, including our *Mycobacterium* genus-specific primers Mb235 (3') and SP1 (5'). Samples were denatured at 95°C for 5 minutes; followed by 40 cycles of the following: 45 seconds at 95°C, 45 seconds at 56°C, 1 minute at 72°C. The final extension step of 10 minutes at 72°C completed PCR amplification.

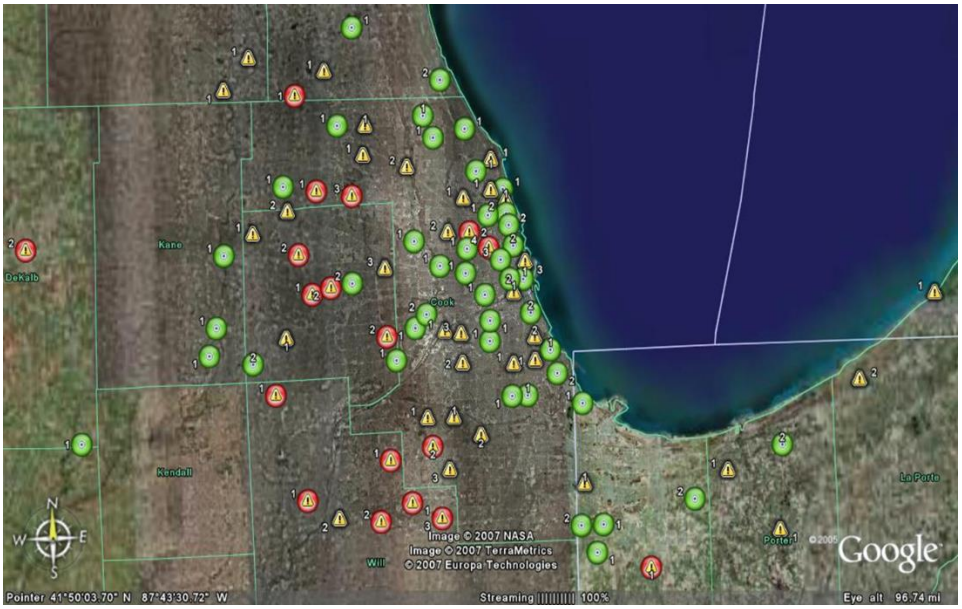

***Figure 7**: Chicago-area water collection areas. A green circle denotes locations with no amoeba presence, yellow triangles denote locations with amoeba presence but no Acanthamoeba presence, red circles indicate areas of Acanthamoeba presence.*

Samples were then run on a 1% agarose gel, made using standard procedure, and each gel was loaded in the following manner: wells 1 through 5 contained the PCR-amplified samples, well 6 contained the positive control (*M. bovis*), well 7 contained the DNA ladder, and well 8

contained the negative control (dH_2O). Gels were run at 120V, for approximately 25 minutes. Gels were subsequently stained with Ethidium Bromide (EtBr) to visualize the DNA bands under UV light. Those bands that were of correct position and intensity as that of the positive control, indicating correct DNA size and amount, were termed "PCR positive for *Mycobacterium*". Bands that were of the correct position and intensity of that of the previously determined *Microbacterium*, were termed "PCR positive for *Microbacterium*". A subset of those samples, as a result of both limited time and limited resources, deemed PCR positive for either bacterial genera were band-isolated and sent for sequencing. Those that were sequence confirmed as being either *Mycobacterium* or *Microbacterium* were termed "Sequence-confirmed".

Results

Of the 36 water samples we screened for the presence of additional intracellular bacteria, 2 samples (6%) contained all 4 bacterial genera (i.e. *Mycobacterium*, *Microbacterium*, *Legionella*, and *Pseudomonas*); 11 samples (31%) contained some combination of 3 bacterial genera; 12 samples (33%) contained some combination of 2 bacterial genera; 11 samples (31%) contained 1 bacterial general. Of the 50 AK samples we screened for the presence of additional intracellular bacteria, 5 samples (10%) contained all 4 bacterial genera; 4 samples (8%) contained some combination of 3 bacterial genera; 14 samples (28%) contained some combination of 2 bacterial genera; 13 samples (26%) contained 1 bacterial genera; 14 samples (28%) did not contain any discernable bacterial genera, from our selected pool of intracellular/endosymbiotic suspects. In total, of the 86 total DNA samples we screened for the additional presence of *Mycobacteria* and *Microbacteria*, 7 samples (8%) contained all 4 bacterial genera; 15 samples (17%) contained some combination of 3 bacterial genera; 26 samples (30%)

contained some combination of 2 bacterial genera; 24 samples (28%) contained only one bacterial genus; and 14 samples (16%) did not contain any discernable bacterial genera, from our selection. **Table 2** shows a summation of our results.

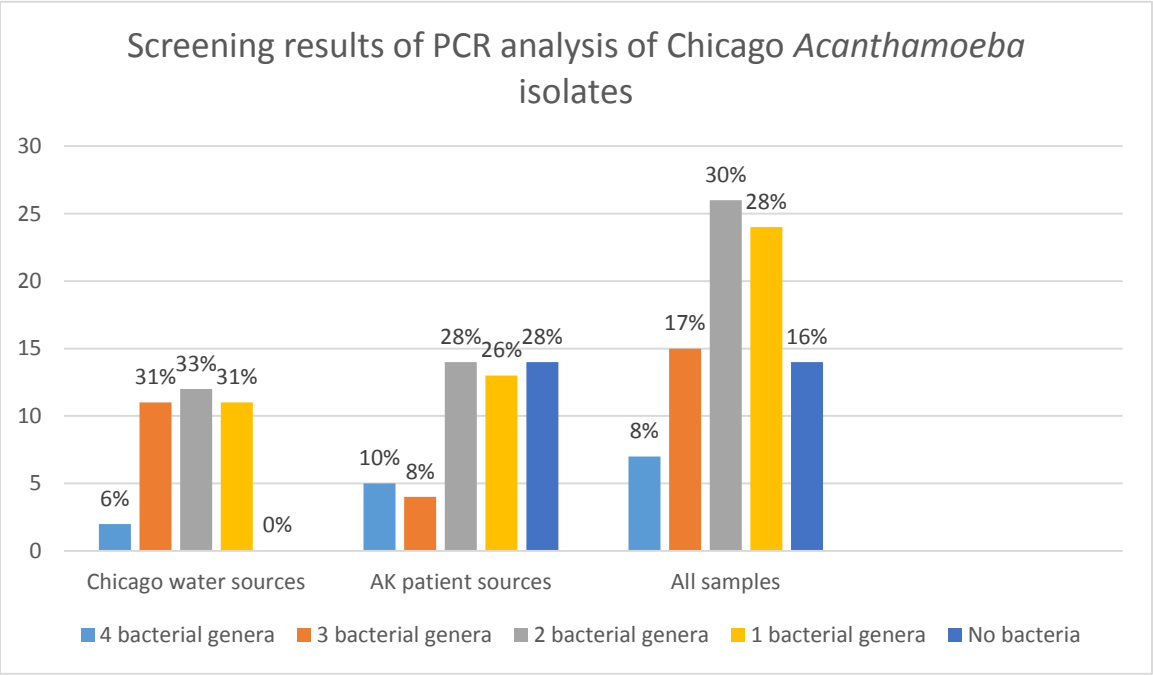

***Table 2**: PCR analysis of Chicago water sources, Chicago AK patients, and the total results of both sample groupings combined.*

Discussion

Here we demonstrate a presence of intracellular bacteria in these Chicago-specific *Acanthamoeba* isolates. This protist is a well-characterized harbor for pathogenic endosymbionts, as *Acanthamoeba* specifically has the ability to protect its intracellular bacteria from numerous antibiotic drugs, all of which these endosymbionts would otherwise be susceptible to.

One way that intracellular pathogens are said to increase the virulence of *Acanthamoeba*-related diseases is in the immune response of the host organism. When *Acanthamoeba* infects a human, the body temperature of that individual is higher than that of where *Acanthamoeba*

trophozoites are typically comfortable. Thus, this increase in temperature that the amoeba experiences during infection of corneal tissue (in the case of AK), would cause the amoeba to encyst and release its intracellular pathogens during the encystment process. These released bacteria would then, subsequently, enhance the immune response in the host organism, leading to a more severe phenotype[37]. Thus, it is our conclusion that the Chicago AK outbreak was a result of the Chicago *Acanthamoeba* harboring intracellular pathogenic bacteria, which may have increased either the virulence of the amoeba, or the process of living inside the amoeba increased the virulence of the bacteria, resulting in a higher level of pathogenicity of the bacteria, and increasing the innate immune response in the host organism's cornea.

Future Directions

In order to fully analyze our samples, we still need to perform sequence analysis of those samples that have only been deemed PCR positive for either bacterial genera. Additionally, an interesting approach may be to use a universal 16S bacterial primers to ascertain any and all bacterial species that may be residing intracellularly, rather than the genus-specific primers which we utilized. While this venture may be both more costly and time consuming, it would conclusively show additional intracellular bacteria found inside these samples at the time of their isolation. This may provide additional insight into the mechanism of phagocytosis, in regards to weakening of the amoeba following the ingestion of pathogenic bacteria (i.e. does the presence of intracellular *Legionella* make *Acanthamoeba* more susceptible to harboring other bacterial species, etc.). Furthermore, an additional interesting venture may be to correlate the number of *Acanthamoeba* endosymbionts in a particular sample to the virulence of that case of AK.

Currently, this type of study would require information regarding patient symptoms that our lab does not have. However, a collaboration may prove fruitful here.

Chapter 3

Mid-Ohio Legionnaire's Disease Outbreak of 2013

From July to August of 2013, a retirement community in Franklin county Ohio experienced a devastating Legionnaire's disease outbreak. The first report of symptoms occurred on July 9th, followed by two additional cases being reported that following Thursday, July 11th [46]. Following the outbreak, there were 39 confirmed cases of Legionnaire's disease linked to this specific outbreak, comprised of residents, visitors, and an employee[14]. From this outbreak, 6 individuals passed away, resulting in this outbreak as being classified as Ohio's largest and deadliest Legionnaire's disease outbreak to date[14].

Due to the severity of the outbreak, the Center for Disease Control and Prevention (CDC) was called in to investigate. Additionally, the retirement community hired an independent contractor to perform both hyper-heating and hyper-chlorination of the water sources used in the nursing home. This included using bleach 25 times stronger than the standard amounts used in tap water[83]. Additionally, as Legionnaire's disease spreads via aerosolization of *Legionella*-contaminated water, the nursing home administration prohibited hot showers, and handed out bottled water, in response to the outbreak[46]. Tests performed by the CDC confirmed the presence of *Legionella* in water that was used for drinking, showering, and cooking, as well as in an air conditioning cooling tower. Furthermore, genotypic analysis confirmed the *Legionella* in the water were genetically identical to the *Legionella* isolated from a patient diagnosed with Legionnaire's from the retirement community[14].

The 2013 Legionnaire's disease outbreak is only one in a string of recent outbreaks. According to the Ohio Department of Health, the number of Legionnaire's disease outbreaks has doubled 2012, and 20% of cases of the disease that have been in Ohio have been in Franklin county.

As our Chicago *Acanthamoeba* keratitis samples indicated, *Legionella* is able to reside inside *Acanthamoeba*, avoiding degradation and treatment. This has been found to occur in both environmental isolates as well as clinical samples of the amoeba. Furthermore, it has been shown that *Legionella* ingestion by *Acanthamoeba* is a necessary step in the virulence of *Legionella*. Prior to completing the encystment process, *Acanthamoeba* releases its intracellular pathogens in vesicles[3]. These vesicles may subsequently be inhaled by host organisms. It has, additionally, been shown that these *Legionella* that have passed through *Acanthamoeba* cause a more severe phenotype than *Legionella* that have been cultured without the presence of *Acanthamoeba*[13].

Materials and Methods

We obtained 21 unfiltered bulk water samples from the CDC, coming from such locations as cold kitchen taps, hot kitchen taps, hot hand sinks, hot master baths, hot bathroom showers, hot water tanks, cooling tower basement reservoirs, and hot water storage towers. These samples were each contained in 50 ml conical tubes. Each sample was filtered using standard procedure, onto a 0.45 Type HA filter paper. One-fourth of the filter paper was plated, face down, onto Non-Nutrient Amoeba Saline (NNAS) Agar plates, with 50 µl heat-killed *E. coli*, and grown at room temperature.

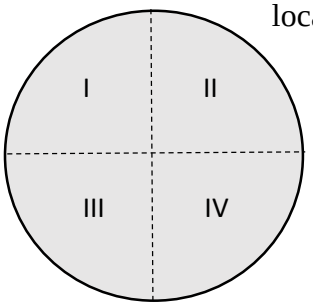

***Figure 8**: Filtered bulk water samples, were divided as shown above. Section I was plated "face down" onto the agar plate, section II was plated "face up", while sections III-IV we left intact and returned to the conical tube.*

We also obtained 19 filtered bulk water samples from the CDC, coming from the same locations as the unfiltered samples, but having already been filtered prior to shipment to our lab. Approximately 950 ml of each 1 L samples was filtered by the CDC, and the filter paper was then submerged in 5 ml of sterile water in a 50 ml conical tube. As we were unsure which side of the filter paper was "up" and which was "down", the filter was cut in half. One semi-circle was returned to the conical tube, and the other semicircle was divided again – one half was plated facing up, the other half plated facing down (**Figure 8**). Filters from the same sample were plated on the same NNAS Agar plate with 50 µl heat-killed *E.coli*, and grown at room temperature.

Lastly, we obtained 9 biofilm swab samples from the CDC. These were contained in 10 ml conical tubes, complete with both the swab, as well as a small sample (approximately 2-3 ml) of water taken from the site of the swab. These were vortexed for 1 minute during the sample processing time, as completed by the CDC prior to shipment. We vortexed each sample for approximately 5 seconds upon arrival to our lab prior to plating, in order to loosen any amoeba or other microorganism that may be present in the sample. 100 µl of each sample was plated on NNAS Ager, with 50 µl of heat-killed *E. coli*, and grown at room temperature.

Each culture plate was scored twice a week for the presence of *Acanthamoeba* by visual analysis using a light microscope. Specifically, we looked for the characteristic autofluorescence of both *Acanthamoeba* trophozoites and cysts, the quintessential "spiky" appearance of the trophozoites, or the clusters of double-walled polygonal-shaped cysts.

Those samples that appeared to contain possible *Acanthamoeba* growth underwent culture splitting. A small cutting of agar was taken from the cultures of interest, and were resuspended in 5 ml Amoeba Saline (AS) with 20 µl heat-killed *E. coli*, in a 20 ml culture flask, and grown at room temperature. In order to sequence analyze the identity of the amoeba we

found in culture, we performed DNA extraction via the protocol found in DNeasy Blood and Tissue Handbook, July 2006. The isolated DNA underwent PCR reaction and gel electrophoresis using standard protocol and *Acanthamoeba*-specific 18S rDNA partial primers, IDP1 and IDP2. *Acanthamoeba* presence was confirmed using DNA sequence analysis.

Once *Acanthamoeba* presence was confirmed, samples that were both sequence-confirmed as containing *Acanthamoeba,* and those samples which we believed to likely be positive for *Legionella* growth (i.e. cooling water tower, large reservoirs of water, etc.) were screened for the presence of *Legionella* DNA using PCR and gel electrophoresis analysis, using *Legionella*-specific primers. Those samples that were PCR positive for *Legionella* underwent band isolation, and sent for DNA sequencing.

Following confirmation of the presence of both *Acanthamoeba* and *Legionella*, the next step was to determine if *Legionella* was physically residing within the amoeba. To do so, we performed fluorescence *in situ* hybridization (FISH) analysis. 3 µl of the AS/*E.coli*/*Acanthamoeba* solution obtained from the culture flask having been shown to contain *Acanthamoeba* trophozoites was spun down and pelleted in a 5 ml tube at low speed, so as not to destroy the amoeba, for approximately 5 minutes. The top liquid layer was discarded in order to isolate the solid *Acanthamoeba* pellet, which was then resuspended in AS. 20 µl of the resuspended *Acanthamoeba* sample was spread on 3 poly-lysine glass slides, and incubated at 45°C for 20 minutes. Slides were washed, fixed using 20 µl of 4% paraformaldehyde, washed again using 1x PBS, and dehydrated using ethanol. 2 polylysine slides were incubated with a GFP-tagged, *Legionella*-specific primer, and 1 slide was incubated with only hybridization buffer (no probe). Owing to *Acanthamoeba*'s autofluorescence, the third slide was not incubated with the fluorescent tag, in order that it may be used as a negative control. Slides were

subsequently washed with washing buffer, again with distilled water, and left to dry at room temperature. Slides were then mounted with mounting media, and examined using a confocal microscope.

Results

To date, only one of our water samples (2%) has been shown to be positive for both *Legionella* and *Acanthamoeba* (**Table 3**). Both samples have been sequence-confirmed by a third party. The sample that was positive (OSU ID 13-018) for *Acanthamoeba* genotype T4 (**Figure 9B**), and *Legionella pneumophila* (**Figure 9A**), was taken

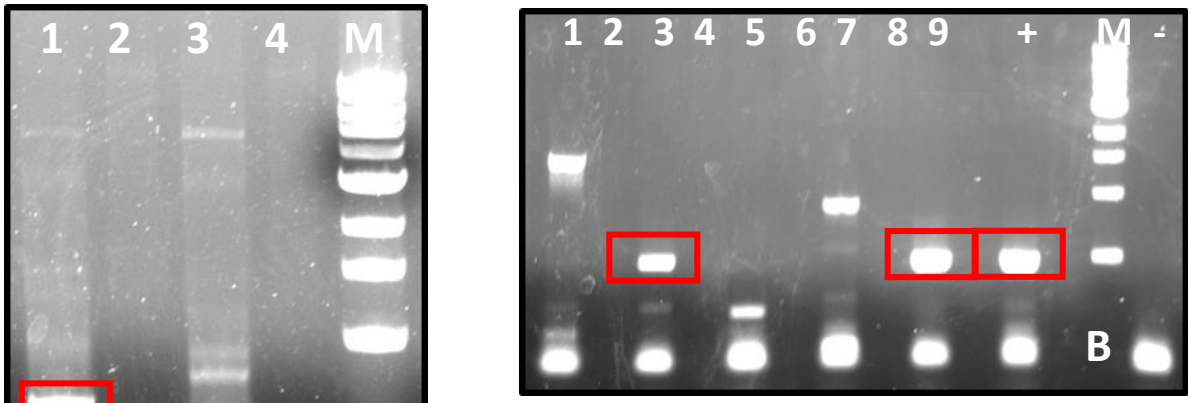

***Figure 9**: PCR results of selected biofilm swab samples. **A** (left) - Analyses for the presence of Legionella in sample 13-018 from swab extraction (lane 1) and in culture (lane 3) using Legionella-specific primers for the 16S gene, as compared against out DNA ladder (lane 3). The red box indicates the correct position and size of Legionella DNA. **B** (top) - Analysis for the presence of Acanthamoeba DNA. Lanes 3 and 9 derive from sample 13-018, from swab extraction and culture extraction, respectively, as indicated by red boxes. Primers were Acanthamoeba genus-specific for the 18S rDNA gene.*

Type of Sample	Total number of samples	*Acanthamoeba*-positive	*Legionella*-Positive*
Biofilm Swab	9	1	1
Unfiltered Bulk Water	21	0	n/a
Filtered Bulk Water	19	0	n/a

***Table 3**: Culture and DNA sequencing results. To date, one sample (OSU ID 13-018) has been shown to be positive for both Acanthamoeba and Legionella. *Only 5 out of 49 samples have been screened for the presence of* Legionella *DNA.*

from a cooling water tower, which is a location that is known to typically harbor *Acanthamoeba.*

Lastly, at the time of this writing, our *in situ* FISH analysis to determine the presence and localization of *Legionella* within the amoeba have been inconclusive, and tests are still underway.

Discussion

As Legionnaire's disease is spread through aerosolization of water contaminated by *Legionella*, it may be possible that the *Acanthamoeba* found in the cooling water tower was able to act as a vector, allowing for the bacteria to be protected from typical water treatment standards. *Acanthamoeba* has the ability to withstand extreme conditions, including temperature, pressure, bleach, and antibiotic treatment. When *Acanthamoeba* ingests *Legionella* via phagocytosis, *Legionella* is able to avoid its own degradation by a protein secretion system that blocks the fusion of the *Legionella*-containing vacuole (LCV) with the lysosome. In this manner, *Legionella* may live, indefinitely, within *Acanthamoeba*, successfully avoiding desiccation by antibiotic treatment and harsh environmental factors. *Legionella* may also proliferate inside the amoeba, lysing its host cell in the process, to infect other host cells.

Had our hypothesis been entirely correct, we may have found more *Acanthamoeba* in our samples, rather than the amoeba being localized to one cooling water tower. However, that being said, we have no insight into the plumbing system and how water is trafficked into the retirement community, thus it is possible that the single source of *Acanthamoeba* was enough to maintain *Legionella* presence and growth in the nursing home's water supply. Furthermore, *Acanthamoeba* is most commonly found in locations comprised of stagnant water. Ergo, a

cooling water tower is considered a relatively typical location to find *Acanthamoeba*, if the genera is present at all.

Thanks to modern medicine and modern diagnostic tools, Legionnaire's disease is not as frightening as it once was considered. That being said, more information regarding the specific mechanism of LD infection is still needed before these disease outbreaks can be mitigated. Every year, between 8,000 and 18,000 people contract the disease and are hospitalized for it, the majority of whom are the elderly, or those with previously compromised immune systems[31]. More recently, there have been at least 21 Legionnaire's disease outbreaks, spanning the globe[86].

Since *Legionella* are able to both reside and replicate inside *Acanthamoeba*, the host cell may act as a possible vector, allowing the bacteria to both grow and evolve in such a way that unprotected growth outside of a host cell would, in all likelihood, disallow. Thus, not only does *Acanthamoeba* harbor these pathogenic bacteria and allow for the evolution into antibiotic-resistant "superbugs", but the amoeba also aids in the transmission of these superbugs from the environment into human and animal hosts[43].

Future Directions

As only one of our samples, OSU ID 13-018, has shown to be positive for both *Acanthamoeba* and *Legionella* presence, it may be interesting, as also considered in the previous chapter, to use universal 16S bacterial primers in PCR analysis to determine what else, besides these two species, may be present within that sample. This could shed light on the capacity of *Acanthamoeba* to harbor a broader array of intracellular bacteria and microorganisms. Also, we will be continuing screening our samples for the presence of *Legionella* bacteria in the rest of our samples, as only 5 out of 49 of our samples have been screened thus far. Further, successful

completion of our FISH analysis to conclusively demonstrate the presence of *Legionella* spp. within the *Acanthamoeba* would bolster our current results and conclusions. Additionally, it may be important to analyze the disease phenotype of the different individuals afflicted when the initial outbreak occurred. Genotypic analysis could provide insight into the severity of the disease experienced, i.e. those who were infected by *Legionella* that passed through the *Acanthamoeba* in the cooling water tower versus those *Legionella* that were never housed intracellularly.

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